# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 Identification of polymorphic site in DNA sequence of the *amh* gene

All steps beginning with the PCR were repeated from the 96 individuals comprised 3 families including each dams and sires to carry the new allele. The *amh* gene length has 3096 bp with start codon AAC. *Amh* gene contains 7 exons that were divided by 6 introns but no splice form similar as *amh* gene structure in Poonlaphdecha *et al*. (2011). The sequences of the DNA fragments were trimmed and SNPs were trimmed and SNPs were manually identified using the program software suit DNASTAR Lasergene 6 (DNASTAR, Inc., Germany) compare with *amh* sequence reference accession no. EF512167.

## 4.2 Anotation of the *amh* variants

After sequencing, the anotation of the *amh* gene of three temperature-treated genetically female (XX) families (03,07and 10) of Nile tilapia showed three single nucleotide polymorphisms (SNPs). Each SNP has 2 types of genotype as homozygous and heterozygous (**Figure 4.1**). SNP1 (G>C) were located in exon 6, SNP2 (G>A) were located in intron, and SNP3 (C>T) were located in exon 7. The genotypes of SNP1 were GC, CC in family03, GG, GC, CC in family07 and GC, CC in family10. SNP2 genotypes were AA, AG in family03, AA, AG, GG in family07 and AA, AG in family10. SNP3 phenotypes CC, CT in family03, CC, CT in family07 and CC in family10 in **Table 4.1**.

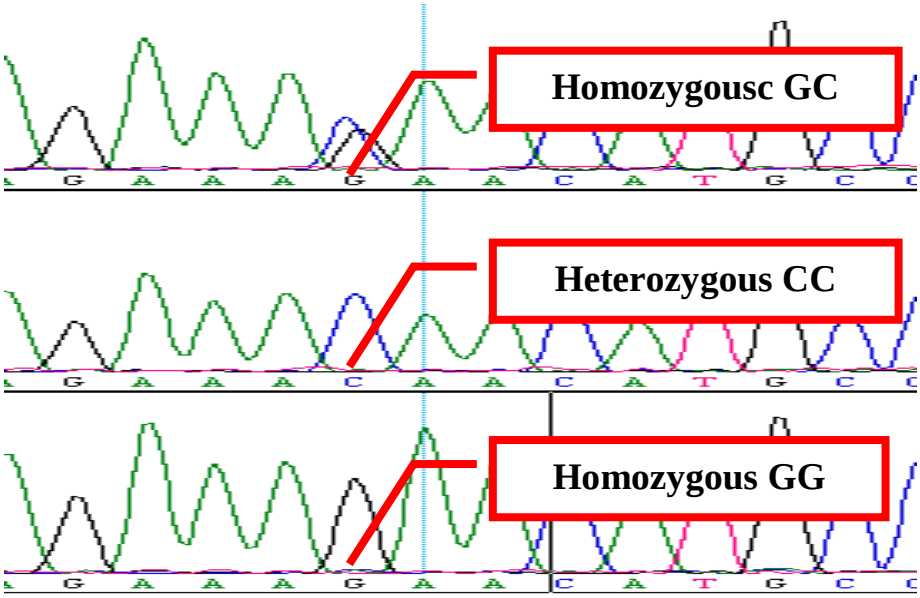



**Figure 4.1** The genotype of *amh* gene in Nile tilapia

**Table 4.1** Observed SNP genotypes in three temperature-treated genetically female (XX) families of Nile tilapia

| | Position | Family 03 | Family 07 | Family 10 |
|---|---|---|---|---|
| SNP1 | Exon 6 | G/C | G/G | G/C |
| | | C/C | G/C | C/C |
| | | | C/C | |
| SNP2 | Intron 6 | A/A | A/A | A/A |
| | | A/G | A/G | A/G |
| | | | G/G | |
| SNP3 | Exon 7 | C/C | C/C | C/C |
| | | C/T | C/T | |

The nucleotide mutation of investigated SNP1 located at position 1899 (G>C) and SNP3 located at position 2708 (C>T) might cause protein function change due to the substitution of amino acid from glutamine to glutamic acid (codons Gaa/Caa) and alanine to valine (codon gCg/gTg) (**Figure 4.2**, **Table 4.2**). Moreover, the mutations of investigated SNPs were located in exons 6, intron 6 and exon 7 and this results might associate with the difference between male and female Nile tilapia. However, the functional roles of the identified SNPs remain to be elucidated**.**

**Table 4.2** The genotypes, codon change and position of three SNPs.

| **SNP** | **Allele** | **Position** | **Codon change** | **Position (in cDNA)** |
|---|---|---|---|---|
| SNP1 | G/C | Exon 6 | ggC/ggG | 1899 |
| SNP2 | A/G | Intron 6 | - | - |
| SNP3 | C/T | Exon 7 | gCg/gTg | 2708 |

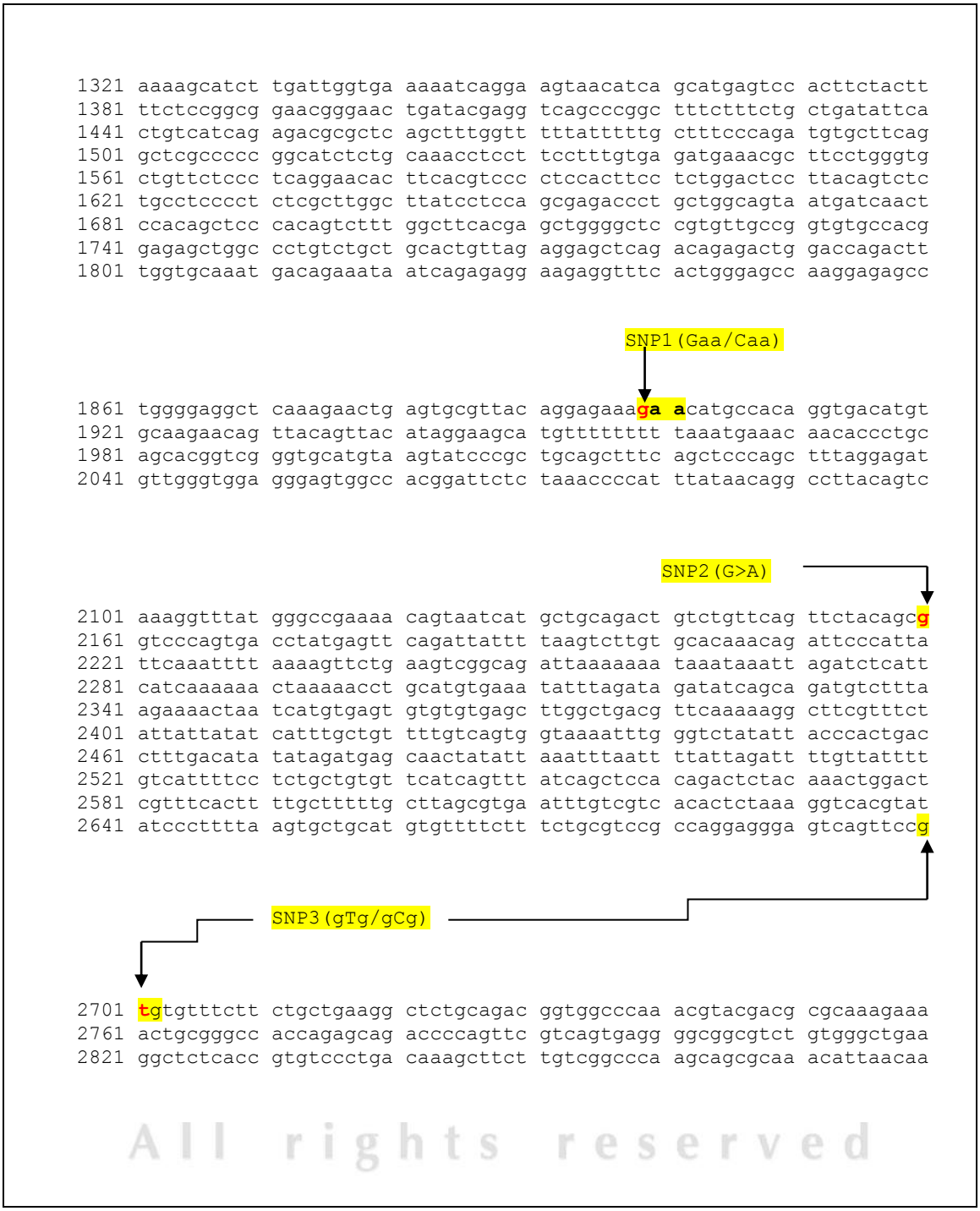
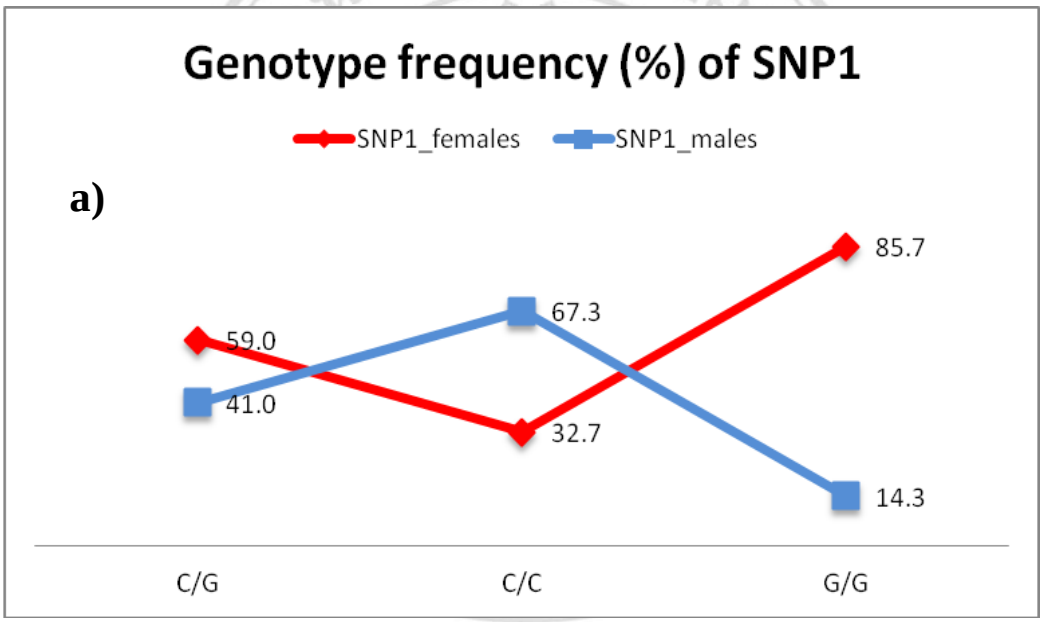
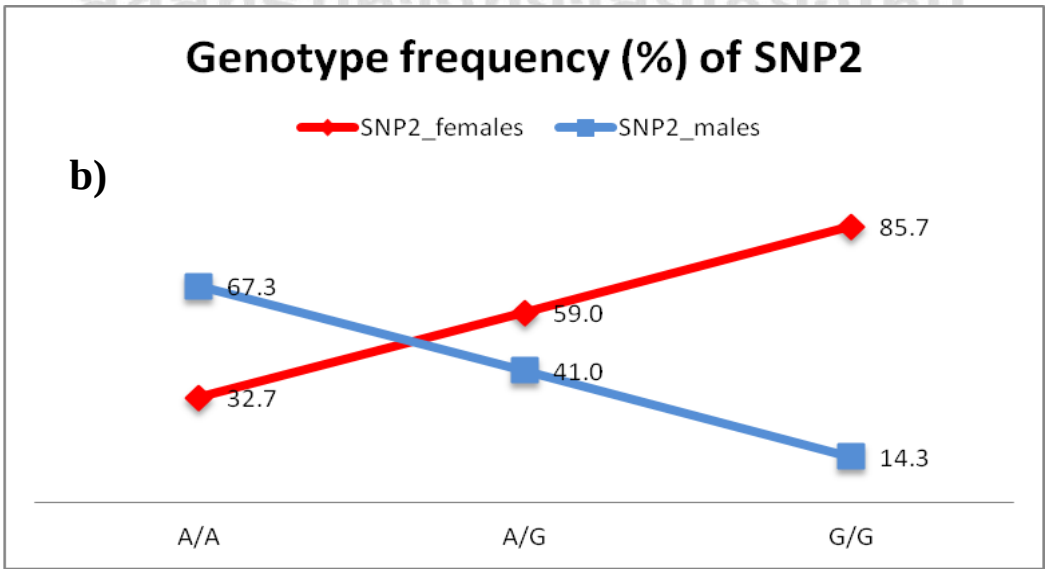



**Figure 4.2** Nucleotide sequences of Nile tilapia *amh* gene from accession no. EF512167.1 (Source: http://www.ncbi.nlm.nih.gov/).

### 4.3 SNPs genotype frequencies

In **Figure 4.3** showed the SNPs genotype frequencies in temperature-treated genetically female (XX) families of Nile tilapia. The SNPs genotypes effect to sex phenotypes divided to male and female. Genotypes CC of SNP1 showed the highest genotype frequency of 67.3 % in male but in female (CC) was lower (32.7%). In SNP2, Genotype AA of 67.3% frequency was higher than female (32.7%). In SNP3, genotype frequency (CC) in male was 61.9% more than female genotype (38.1%). In addition, the other genotype frequencies of three SNPs as GC, GG in SNP1, GA, GG in SNP2 and CT, TT in SNP3 also described.



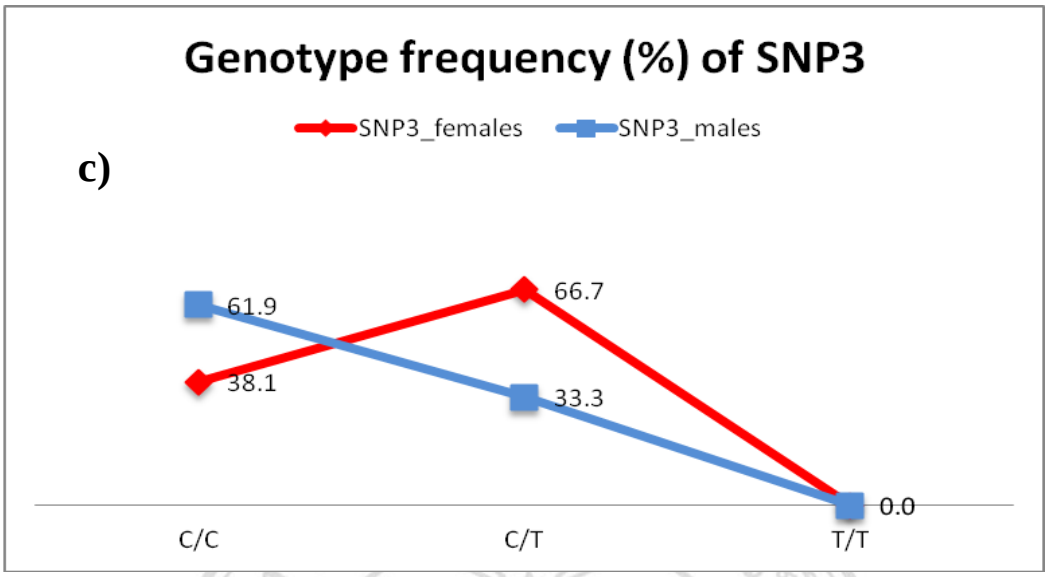



**Figure 4.3** The percentage frequencies of sex phenotype of temperature-treated Nile tilapia are represented by graph, red line showed sex genotype of females fish and blue line showed sex phenotype of males fish a) SNP1 sex genotype frequency b) SNP2 sex genotype frequency and c) SNP3 sex genotype frequency.

### 4.4 Association between SNPs and sex phenotype

The chi-square analysis was used as the statistic program to determine the interaction between 3 SNPs and sex phenotype. The results of chi-square analysis of SNP1, SNP2 and SNP3 were 0.007, 0.018 and 0.010, respectively ($P<0.05$). The results indicated that the genotype frequencies in 3 SNPs of temperature-treated Nile tilapia had significantly association with the sex phenotype. The results were showed in **Table 4.3**

**Table 4.3** The effect of three SNPs genotype frequencies and their interaction on the sex phenotype of temperature- treated Nile tilapia

| | Population (N) | Genotype frequencies | | Chi-square | P-value (95%) |
|---|---|---|---|---|---|
| | | Male | Female | | |
| SNP1 | 93 | 67.3 (CC)<br>41.0 (GC)<br>85.7 (GG) | 32.7 (CC)<br>59.0 (GC)<br>14.3 (GG) | 0.007 | <0.05 |
| SNP2 | 93 | 67.3 (AA)<br>41.0 (GA)<br>85.7 (GG) | 32.7 (AA)<br>59.0 (GA)<br>14.3 (GG) | 0.018 | <0.05 |
| SNP3 | 93 | 61.9 (CC)<br>33.3 (CT)<br>0.0 (TT) | 38.1 (CC)<br>66.7 (CT)<br>0.0 (TT) | 0.010 | <0.05 |

### 4.5 Linkage disequilibrium (LD)

The 3 SNPs (SNP2, SNP2 and SNP3) segregating were investigated in haplotypes correlation as Linkage disequilibrium (LD) of 93 individuals include their parents. The LD was calculated in $r^2$-value by comparing two SNPs. The results showed that SNP1 and SNP2 were full linkage disequilibrium (LD) (0.9996)**.** The LD between SNP1 and SNP3 as well as SNP2 and SNP3 showed equal of 0.54 in $r^2$-value **(Table 4.4).** In addition, the SNP2 (intron6) was not considered as the potentially causal SNP due to the position of SNP2 was in non-coding region. However, the role of $r^2$-value from 0.5-1.0 could indicate the association between comparing of 2 or more then 2 factors. Therefore, in 3 SNPs especially SNP1 which had the $r^2$value 0.54 and full LD (~1) had the association between each as haplotype and it could be the cause of sex differentiation in temperature-treated Nile tilapia

**Table 4.4** The analysis of linkage disequilibrium (LD) in *amh* gene detected in temperature-treated genetically all-female (XX) Nile tilapia

| | **Popula-tion (N)** | **Disequilibrium coefficient (D)** | **Normalized disequilibrium coefficient (D')** | **Coefficient of determination ($R^2$-Value)** |
|---|---|---|---|---|
| LD (SNP1,SNP2) | 93 | 0.2036 | 0.9996 | 0.9996 |
| LD (SNP1, SNP3) | 93 | 0.0895 | 0.7765 | 0.5394 |
| LD (SNP2,SNP3) | 93 | 0.0895 | 0.7765 | 0.5394 |